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Behavioral and genomic characterization of molt-sleep in the tobacco hornworm, *Manduca sexta*

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ABSTRACT

During the transition from feeding to molting, larval insects undergo profound changes in behavior and patterns of gene expression regulated by the neuroendocrine system. For some species, a distinctive characteristic of molting larvae is presence of a quiescent state sometimes referred to as "molt-sleep". Here, observations of 4th instar *Manduca sexta* larvae indicate the molting period involves a predominantly quiescent state that shares behavioral properties of adult insect sleep in that it is rapidly reversible and accompanied by a reduced responsiveness to both mildly arousing and noxious stimuli. When subjected to noxious stimuli, molting larvae exhibit locomotory and avoidance behaviors similar to those of inter-molt larvae. Although less consolidated, inter-molt quiescence shares many of the same behavioral traits with molting quiescence. However, when subjected to deprivation of quiescence, inter-molt larvae display a compensatory rebound behavior that is not detected in molting larvae. This suggests that molting quiescence is a specialized form of inactivity that affords survival advantages to molting larvae. RNA-seq analysis of molting larvae shows general reduction in expression of genes encoding GPCRs and down regulation of genes connected with cyclic nucleotide signaling. On the other hand, certain ion channel genes are up-regulated, including transient receptor potential (TRP) channels, chloride channels and a voltage-dependent calcium channel. These findings suggest patterns of gene expression consistent with elevation of quiescent state characteristic of the molt in a model holometabolous insect.

1. Introduction

During juvenile stages, insects feed and grow. Presence of an exoskeleton (cuticle) necessitates periodic molts during the growth phase, which involve synthesis of new cuticle and shedding of the old. The insect molt serves as an excellent model to examine mechanisms through which the neuroendocrine system coordinates behavior with physiology. The rise and fall of molting hormones programs successive bouts of gene expression in epidermal cells necessary for new cuticle synthesis along with digestion and recycling of the old (Riddiford, 1986). Molting hormones also target the central nervous system and endocrine glands to program ecdysis behavior—the centrally generated stereotyped behavioral sequence that leads to shedding of undigested cuticle at the end of the molt (Zitnan et al., 2007; Zitnan and Adams, 2013). Failure to perform ecdysis results in the confinement of the animal in the old cuticle and prevention of further growth.

The *absence* of motor behavior at the appropriate time is also an important component of an animal's behavioral repertoire. Resting behavior may contribute to fitness through a variety of means, including recovery of motor function, energy conservation, and avoidance of predation. Sleep-like states often are associated with more prolonged periods of inactivity and differ from rest in that they are characterized by reduced sensory responsiveness (Campbell and Tobler, 1984). Sleep states also can be differentiated

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from comatose states or inactivity due to motor impairment, in that they are rapidly reversible—strong stimuli can arouse the animal, and woken animals respond appropriately to subsequent stimuli. Functions of sleep and sleep-like states are of intense interest, due to their widespread occurrence across animal phyla and observations that sleep deprivation can lead to cognitive and motor impairments and in extreme instances, even death (Rechtschaffen et al., 1989; Shaw et al., 2002; Tobler, 2000).

Adult *Drosophila melanogaster* became an important invertebrate model system for the study of sleep following findings that its period of consolidated nighttime inactivity exhibits sleep-like properties of reduced responsiveness, rapid reversibility and homeostatic regulation (Hendricks et al., 2000; Shaw et al., 2000). Although the search for molecular correlates of sleep-states has largely focused on the central nervous system (the brain in particular), involvement of the endocrine system is suggested by findings that the steroid molting hormone has a sleep-promoting role in the adult fly (Ishimoto and Kitamoto, 2010). Presence of sleep-like states during larval stages of insects has not been investigated, but in the developing nematode *Caenorhabditis elegans*, periods of behavioral quiescence or "lethargus" during larval molts display sleep-like behavioral properties similar to those identified in flies (Raizen et al., 2008).

Larvae of *Manduca sexta* reportedly exhibit prolonged periods of inactivity or quiescence during the molt (Reinecke et al., 1980). Such quiescence has (through casual observation) been termed the "molt-sleep", but detailed knowledge of this behavioral state remains limited. Under optimal conditions, *M. sexta* develops through five larval stages, and the quiescent period immediately preceding ecdysis to the 5th and final larval has been suggested as lasting for over a day (Reinecke et al., 1980). Accessibility (unlike *Drosophila* larvae, it does not burrow), and relatively large nervous system and hemolymph volume has aided discovery and characterization of hormones that govern the molt, as well as behavioral and electrophysiological studies that have helped define ecdysis behavior. Since the reported timing of molting quiescence places onset of the so-called molt-sleep sometime during or following the surge of molting hormone, it is possible that in addition to their peripheral actions on the epidermis and central actions that program ecdysis behavior, molting hormones also may effect the nervous system—either directly or indirectly—to cause the onset and maintenance of the molt-sleep. During pupation and metamorphosis, molting hormones act on the central nervous system to bring about remodeling of circuitry, but this remodeling is reportedly absent during the larval–larval stage transitions (Booker and Truman, 1987; Granger et al., 1989; Homberg and Hildebrand, 1994). It is possible that quiescence during the molting period may be due to motor deficits attributable to structural changes in the periphery. However if motor functions remain intact, then it is likely that molting quiescence results from changes in central neuron activity that affect motivational state, in which case the larval molt would offer an excellent model system for studies of neuroendocrine regulation of insect arousal states, including possible sleep-like states.

Here we compare rest-activity profiles of inter-molt (feeding) and molting stage larvae and clarify the timing of behavioral transitions in relation to predicted neuroendocrine events. By challenging larvae with both mildly arousing and noxious stimuli, we demonstrate that although quiescence is accompanied by a reduced sensory responsiveness, peripheral changes associated with the molt do not impair normal motor function. Once aroused, molting larvae respond to stimuli as robustly as do inter-molt larvae. Although larval quiescence during both inter-molt and molt phases share sleep-like properties of reduced responsiveness and reversibility, only inter-molt larvae show compensatory increased quiescence following deprivation, suggesting a possible shut-down of homeostatic control mechanisms during the molting period. Finally, the recent availability of the *M. sexta* genome enabled us to report the first larval brain transcriptome for this species. The predictability of behavioral transitions relative to developmental and weight markers allowed us to examine the brain transcriptome during the transition to the molt-sleep. We report thousands of genes predicted to be differentially expressed in the brain relative to the mid-inter-molt feeding stage.

2. Materials and methods

2.1. Insect rearing

Manduca sexta were reared at the University of California, Riverside. Adults were housed at 27 °C under a 16L:8D light cycle and fed 40% sugar water *ad libitum*. All larval stages were reared in 25 °C incubators with 17L:7D light cycles. Eggs were transferred to incubators and hatched 1st instar larvae were placed in individual 1 oz plastic cups and moved to 5 oz cups as late 4th or early 5th instar larvae. Larvae were fed *ad libitum* on a modified wheat germ-based artificial diet (Bell and Joachim, 1976).

2.2. Quiescence profiles

Behavior was monitored in an isolated dark-room at 25 °C using time-lapse digital recordings. A webcam and time-lapse recording software (Flix v3.3; Nimisis) was used to acquire images onto a desktop computer and behavior was analyzed using an imaging capturing/processing utility (Virtualdub v1.9.7). Frames were captured every 10 s and each frame was scored by visually monitoring shifts in position between frames. Frames received a score of "1" if position was unchanged compared to the previous frame (quiescent between frames), and a score of "0" if active between frames. Fractions of quiescence were then calculated for selected time-blocks. Quiescent-bout durations were calculated using Clampfit v10.2 software (Molecular Devices). Quiescent-bout initiation was defined as the beginning of any period of inactivity $\geq$10 s and the termination of a quiescent bout was defined as the beginning of any period of activity $\geq$30 s.

For profiles spanning a larval instar, each stage was recorded independently and individual larvae were used for the recording of one instar only. For 3rd and 4th instars, larvae with fluid filled head capsules were selected and recording sessions began prior to ecdysis to the stage of interest. For 2nd instar profiles, recordings began the day following ecdysis. Larvae were placed in individual arenas constructed from polystyrene Petri dishes with larval diet forming the base of the arenas. Arenas were placed on a vibration isolation table and were shielded from air currents. For maximal resolution, only 3 arenas were monitored during each recording session. Except when indicated, profiles were acquired under continuous light (L:L) conditions (Energy Smart Soft White 13 Watt bulbs #72466, 870 lumins; General Electric). For larvae recorded under a L:D cycle, infrared lighting (wavelength 850 nm) was used to monitor activity during the scotophase period and only one arena was monitored per recording session. For starved controls, methods were the same except that arenas did not contain food, and larvae were staged so that recordings began ~24 h prior to ecdysis (*see staging methods for brain dissections*).

2.3. Arousal thresholds and reversibility

The water bath was set to room temperature (25 °C), 40 °C, or 50 °C. Behavior arenas were constructed using 23 cm diameter

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foam plates (Stater Bros) with 12.5 cm diameter holes cut out of the middle. Holes were covered with filter paper (15 cm diameter; Whatman #1005150), which formed the base of the arena. For "DRY" conditions, filter paper was secured to intact plates. A plastic, two-tiered circular ledge formed a rim around the circumference of the arena. Prior to testing, larvae were placed in the center of an arena under 1.5 oz plastic cups for a ~15 min equilibrium period.

Placement on the water bath coincides with an immediate dampening of the filter paper. Responses were recorded for 10 min or until larvae escaped contact with the damp surface by climbing over the plastic ledge. Frames were captured every 0.2 s and each frame was scored as described above for *quiescence profiles*. The time of stimulus onset was defined as the frame during which the wetting of the filter paper encompassed an area that completely surrounded the larva. Locomotion onset was defined as the frame corresponding to the initiation of peristaltic contractions that led to a shift in larval position. Time of edge contact was defined as the frame where the anterior tip of the larvae made contact with the peripheral ledge. Larvae were considered to have escaped the arena if at any time all portions of the body were lifted from the wet filter paper. For larvae that did not escape under DRY and room temperature wet conditions, quiescence levels were determined using methods outlined in *quiescence profiles*. All larvae were tested only once. Following testing, time-lapse digital recordings were used to determine the time of ecdysis to assure assignment to the correct developmental category. For most comparisons we used late 4th (~40 h prior to ecdysis) and middle-molt (~15 h prior to ecdysis) larvae as they are similar in both size and weight.

2.4. Quiescence deprivation

To deprive larvae of quiescent behavior a semi-automated deprivation system was devised. A cardboard lid with dimensions 27 cm × 27 cm was secured upside-down to a test tube mixer/rocker (Thermolyne Vari Mix #M48725). Within the lid, 3 separate tracks were constructed using 2 ml polystyrene disposable pipets that spanned the area of the lid. Each track was constructed to allow the unimpeded movement of a toy car (2010 Ford Mustang GT Hot Wheels Mattel). Larvae were placed in 5.5 oz plastic cups that were secured to the roof of each toy car. Larval diet formed the base of each cup. Rocker speed was set to 1 rock/12-14 s. During each rock, toy cars bumped into *Drosophila* vial Flugs that had been secured to the lid at the ends of each track. Impact did not result in any visible injury to the larvae. During most rocking cycles, larvae were either dislodged from their position on the food, or rolled so that they were forced into a righting reflex. Larvae were visually monitored so that if they were able to secure themselves against periodic rolling, they were gently dislodged from their position using blunt forceps or by tapping the cup. Larvae that showed visible signs of injury due to handling with forceps were excluded from further analysis. Control larvae were kept in the same room and type of arenas on a table separated from the rocker. Inter-molt larvae were staged so that deprivation was terminated at 9 PM−27 h following ecdysis to the 4th instar. Inter-molt larvae were weighed in the morning on the day of deprivation and again immediately following the termination of deprivation and the percent gain in weight was recorded. Molting larvae were deprived during either the last 6−9 h of the molt to the 4th instar, or during the molt to the 5th instar following head capsule slippage. Immediately following the deprivation sessions, deprived and control animals were transferred to 1.5 oz cups containing larval diet and post-deprivation behavior was recorded and analyzed as described for *quiescence profiles*.

2.5. Staging for dissections

Larvae were staged relative to the time of the head capsule slippage (HCS) occurring during the molt to the 5th instar. Only gate II 4th/gate I 5th instar larvae were used, as these were found to be highly synchronous when staged using weight indicators and developmental markers. For 17D:7L incubators, lights on was set to 8:30AM. Pharate 4th instar larvae were selected only if HCS occurred after 4PM, weights were greater than 200 mg and by 9AM the following morning, larvae had either ecdysed to the 4th instar or showed air-filled head capsules with blackened mandibles. Under our rearing conditions, selected larvae undergo the next HCS ~54−57 h following ecdysis to the fourth stage. For larvae dissected at −18, −12 and −6 h relative to HCS, additional weighing occurred at −22 h; only larvae over 600 mg by −24 h were selected. All larvae dissected at or following HCS were staged by visually monitoring the time of HCS.

Brains were dissected by first chilling larvae on ice until immobile, and then decapitating and pinning whole heads onto a dissection dish filled with ice cold saline made to the specifications of Zitnan et al. (1999). Prior to HCS, brains were exposed by cutting a window through the head capsule using a sharp scalpel. Following HCS, brains were exposed by pinning the head head-capsule side down. Nerves and trachea were carefully trimmed close to the supraesophageal ganglia (brain) and brains were placed in a tube on dry ice. Dissections were completed in under 4 min. Pooled brain samples were stored at −80 °C. For Illumina sequencing, 23-41 brains were collected for each pooled sample. For qPCR, 9-24 brains were collected for each pooled sample (biological replicates) for each stage.

2.6. Ilumina RNA-seq

Total RNA was extracted from pooled brain samples using TRIzol (Invitrogen). Quantity and quality of extracted total RNA was assessed using a Nanodrop ND1000 Spectrophotometer and an Agilent 2100 Bioanalyzer. An average of 9.8 ± 1.7ug (per sample) of high quality total RNA was submitted to Illumina's FastTrack Sequencing Services (Illumina, Inc., Hayward, CA) for further processing and sequencing. Samples were processed according to the Illumina RNA-Seq in-house protocol for the Genome Analyzer sequencing platform (http://www.illumina.com). cDNA fragment size selection was in the range of 150-180bp (insert size). Each sample was run on a single flow cell lane, and single read sequencing was used to sequence each sample to 75 bases.

2.7. qPCR

Total RNA was extracted from pooled brain samples using TRIzol (Invitrogen) and quantity and quality of extracted total RNA was assessed using a Nanodrop ND1000 Spectrophotometer. 1 ug of total RNA was reverse transcribed into cDNA using Superscript First Strand Synthesis System for RT-PCR kit (Invitrogen) according to the manufacturer's protocol. Samples were DNase treated using TURBO DNA-free (Applied Biosystems/Ambion) prior to reverse transcription and no-RT controls were used to check for contamination.

Primers were designed from known *M. sexta* mRNA sequences reported in the NCBI database. Gradient PCRs followed by electrophoresis on 2% agarose w/ethidium bromide gels were first used to check for specificities of primers and optimal annealing temperatures. Sequence identity of each amplicon was verified using Sanger (BigDye) sequencing (Applied Biosystems). Serial dilutions of cDNA were used with qPCR to generate standard curves to evaluate amplification efficiency. QPCR was carried out in 25 ul reaction

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volumes containing 12.5 ul of IQ SYBR Green Supermix (Bio-Rad), 2 ul cDNA template, primers at the optimized concentration, and water. The Bio-Rad iQ5 Real Time PCR Detection System was used according to the manufacturer's specifications. For each biological replicate, cDNA (10-fold dilutions) from specified developmental time-points was run in triplicate on the same plate. For all reactions, cycling parameters were 1 × 3 min @95 °C, 40 × (20 s @95 °C, 20 s @60 °C, 20 s @72C°), 1 × 1 min @95 °C, 1 × 1 min @55 °C, followed by a melt curve from 55 to 95 °C at 0.5 °C steps (10 s/step) to verify amplification specificity. All primers were used at a concentration of 200 nM.

For all profiles, *M. sexta* ribosomal protein L32 was used as an internal reference gene. Control reactions were run in triplicate in parallel with experimental reactions. Results were analyzed using the iCycler iQ Optical System Software v2.0 (Bio-Rad). Thresholds were set manually within the linear amplification range and cycle threshold (Ct) values were exported to Microsoft Excel for further analysis. Relative expression ratios were calculated by the Pfaffl equation ((Pfaffl, 2001).

2.8. Alignment of RNAseq reads to the M. sexta genome

Library reads were aligned to the Msex_1.0 assembly available at the National Center for Biotechnology Information (NCBI) under GenBank Assembly ID GCA_000262585.1. Because an official list of transcripts was not yet available at the time of the analysis, the most up to date set of preliminary transcripts available was used instead. These preliminary transcripts are maintained at the Kansas State University AgriPestBase database (file OGS2_20140407.gff was used for this analysis). Alignment was performed using Tophat2 (Kim et al., 2013) software using the above assembly, transcript, and RNAseq library files.

2.9. Differential gene expression

Differential gene expression analysis between selected pairs of libraries was performed using the DESeq and R package that uses a model based on the negative binomial distribution (Anders and Huber, 2010). Uncorrected number of reads mapping to each gene were obtained using BEDTool utilities (Quinlan and Hall, 2010) and were used as input for DESeq. Working with normalized counts of one pair of libraries at a time, the fold change value for each gene pair was estimated. Each library was compared individually to the designated inter-molt reference library (HCS −30 h). For the purposes of this study, genes were predicted as differentially expressed if fold-change values were >2 or <0.5.

2.10. Sequence annotation

BLAST2GO v.2.7.2 (Götz et al., 2008) was used to perform sequence annotation on a set of 3543 genes predicted as differentially expressed. For each gene, sequence data for the transcript isoform with the highest expression levels were uploaded into BLAST2GO. Using default parameters, nucleotide sequences were blastx-searched against the NCBI nr protein database through QBLAST. For all BLAST hits, the mapping step was used to retrieve associated gene ontology (GO) terms, gene names or symbols, and UniProt IDs (www.geneontology.org); Non-redundant Reference Protein Database). Sequences were annotated using the applications default parameters. Annotation was increased by running the InterProScan search against all EBI databases to retrieve GO terms associated with domain/motif information. Annotations were augmented using the Annex function prior to further analysis.

2.11. Cluster analysis

The assignment of genes into clusters of similar expression profiles was carried out using the software program Short Time-series Expression Miner (STEM) (Ernst and Bar-Joseph, 2006). Normalized read counts for the 3543 genes predicted as differentially expressed were loaded into STEM and log-normalized to the earliest developmental time-point (HCS −48 h). Missing values ("0" counts) were replaced with a value of "1" prior to loading. Profile significance was corrected for multiple testing (Bonferroni correction with a FDR ≤ 0.05). Similar profiles were grouped into clusters based on a correlation coefficient ≥0.7). For all other settings we used the default parameters specified in the user manual (STEM v1.3.8).

2.12. Transcript identification

For transcripts assigned to clusters C1−C8, descriptions and GO terminologies assigned by BLAST2GO were searched for terms related to the functional categories *G-protein coupled receptor*, *cyclic nucleotide signaling pathway*, *neurotransmitter/neuropeptide*, and *ligand-gated ion channel/ion channel*. Putative orthologs were identified using the reciprocal BLAST approach (Fan et al., 2010). Each *M. sexta* sequence was first queried against the NCBI database, the Silkworm Genome Database: SilkDB, and FlyBase using default blastx parameters with an additional Expect value $<10^{-6}$, and the highest scoring sequences from each database were then queried against the current list of *M. sexta* transcripts provided by the AgriPestBase database (Manduca Base). If the highest scoring sequences reciprocally identified the original *M. sexta* sequence as the highest scoring BLAST hit, sequences were predicted to be putative orthologs, implying a similar function and biological role. Descriptions and gene names assigned to putative orthologs were in turn used to describe selected *M. sexta* transcripts.

3. Results and discussion

3.1. Larval rest-activity profiles show developmental rhythms

During larval stages, *M. sexta* displays a variety of behaviors, including subtle head movements associated with feeding and searching, grooming, locomotion, and defensive responses (Bernays and Woods, 2000; Miles and Booker, 2000; Walters et al., 2001). Rest-activity profiles were generated by focusing on "behavioral quiescence", defined here simply as a time interval during which no movement could be detected. Any movement was considered as a generalized state of arousal associated with increased probability of behavior (Pfaff et al., 2008).

Time-lapse digital snapshots were used to monitor 4th instar larval behavior every 10 s, from shedding of the 3rd instar cuticle to shedding of the 4th instar cuticle (Fig. 1A). Under continuous light conditions, duration of the 4th stage varied from 90 to 110 h, indicating that both Gate I and Gate II larvae were observed (Truman, 1972). Although fourth instar inter-molt quiescence levels do not display daily activity fluctuations, our repeated measures analysis revealed that they show significant quadratic decay (LRT = 136.6698, p-value < 0.0001) as the inter-molt period progresses across the multiple days of feeding (Fig. 1B). These results are in agreement with previous studies showing lack of time-of-day influences on levels of feeding behavior or fecal evacuations during inter-molt stages (Bernays and Woods, 2000; Reynolds et al., 1986; Stewart and Nelson, 1977). Based on similar repeated measure analysis, we found that levels of quiescence increase nonlinearly during the period when animals transition to the molt (LRT = 247.1651, p-value ≤ 0.0001) (Fig. 1C). The rise in quiescence

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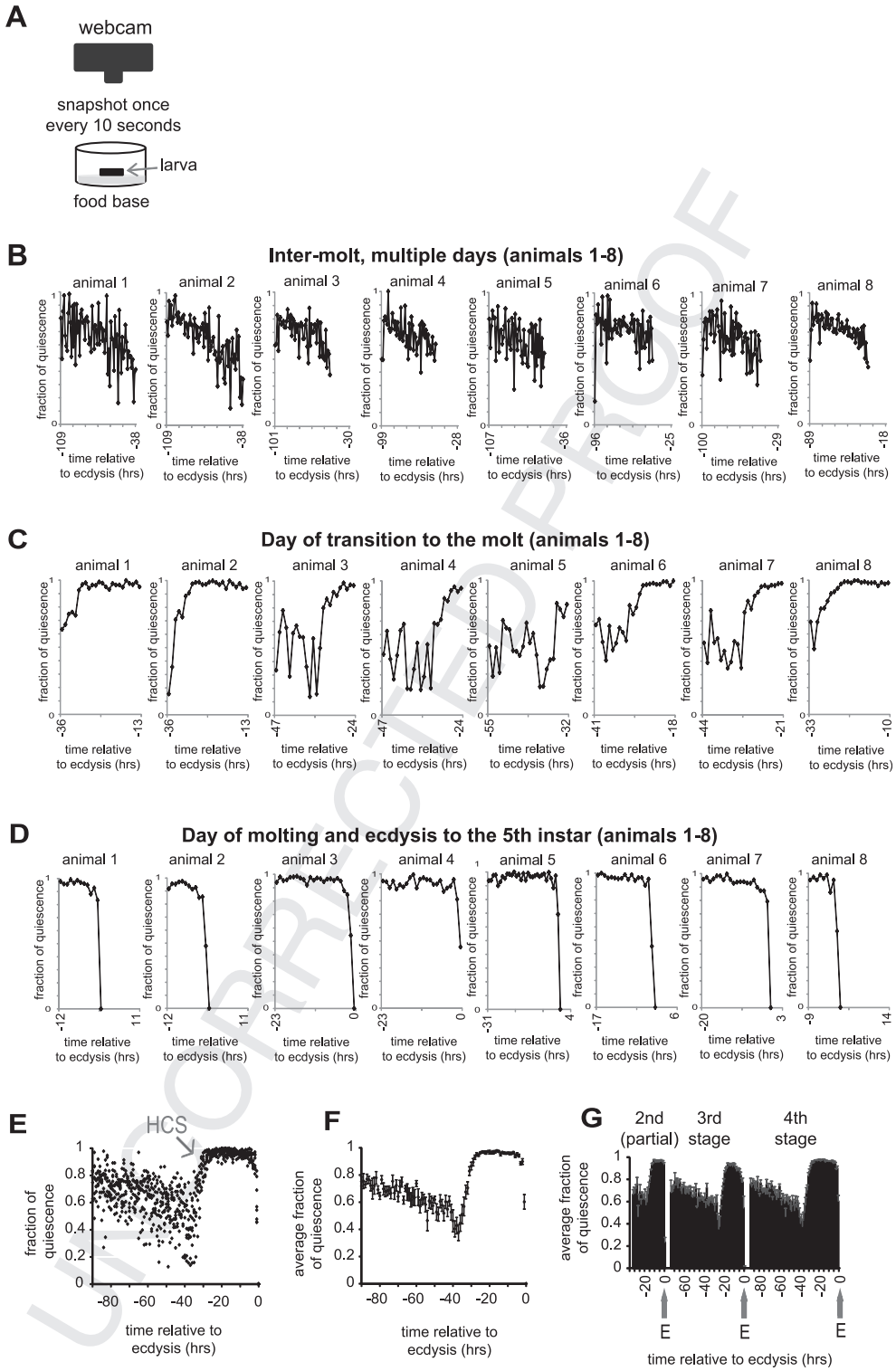

Fig. 1. Larval rest-activity patterns show developmental rhythms. (A) Schematic of recording set-up. (B) Fourth instar inter-molt larval activity recorded under constant light conditions. Shown for each individual animal are fractions of quiescence calculated in 1 h bins, plotted relative to the time of ecdysis to the 5th instar. The final data point in each plot occurred between 11 PM and midnight (time of day). ($N = 8$) (C) For animals in (*B*), fractions of quiescence are plotted relative to time of ecdysis for a 24 h period on the day of transition to the molt. (D) For animals in (*B,C*), fractions of quiescence are plotted relative to the time of ecdysis on the day of molt termination. For (*C,D*), Plots start at fractions that occurred midnight to 1AM. Time "0" indicates time of ecdysis to the 5th instar. (E) For the 8 larvae analyzed in (*B–D*), 1 h fractions of quiescence are plotted relative to the time of completion of ecdysis (time "0) to the 5th instar. Head capsule slippage (HCS) occurs where indicated. (F) Average quiescence levels across all 8 animals set relative to ecdysis. (G) Rest-activity profiles were also recorded under constant light conditions across the 3rd instar ($n = 6$), and for the latter part of the 2nd instar ($n = 6$). Bars are average 1 h fractions of quiescence plotted relative to the time of ecdysis to the next stage. Arrows indicate time of ecdysis completion. All error bars represent (s.e.m).

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level occurs at the time of apolysis (a.k.a "slippage") of the head capsule (HCS)—an event reported as directly preceding onset of the "molt-sleep" ~30 h prior to ecdysis (Reinecke et al., 1980; Truman, 1972). Following HCS, quiescence is maintained at elevated levels with slow quadratic decay (LRT = 40.18773, p-value <0.0001) until ecdysis onset and termination of the molt (Fig. 1D) When fractions of quiescence for all animals are plotted relative to the time of ecdysis to the 5th instar, a distinct developmental pattern emerges that is independent of instar duration (Fig. 1E,F). A comparison of 4th instar quiescence levels among 15 "6 h bins" indicates that quiescence level during the ~30 h molting period is significantly elevated relative to quiescence during the inter-molt feeding stage (Fig. S1A). Larvae recorded under a light–dark cycle have rest-activity patterns similar to those recorded under continuous light (Fig. S1B). Similar developmental patterns were observed for 3rd and 2nd stage larvae (Fig. 1G), indicating a generalized underlying control mechanism.

Timing of head capsule slippage and ecdysis behavior are known to occur at fixed intervals following initiation of endocrine events leading to molt onset (Truman, 1972). We likewise demonstrate that quiescence levels are highly predictable relative to those events. The sudden increase in quiescence level at HCS correlates well with predicted timing of the ecdysteroid surge that programs physiological outcomes of the molt (Langelan et al., 2000), suggesting that molting hormones may also influence larval arousal state.

3.2. Molting quiescence is characterized by an increase in maximal bout duration

Feeding behavior of inter-molt larvae occurs in discreet bouts (Bowdan, 1988; Reynolds et al., 1986). To further our comparison of inter-molt and molt activity-levels, 10 min fractions of quiescence were plotted relative to time of ecdysis and shifted 10 min for each data point for individual 4th stage larvae. Note that downward deflections represent increased activity. When plotted in this way, individual larval profiles show marked reduction of activity bout duration during the molt (Fig. 2A); these changes are accompanied by quiescence bout durations (>1 h) that are nearly absent during the early and late inter-molt feeding stages, as suggested by Fisher Exact tests on 2 by 2 contingency tables of stages (early inter-molt and late inter-molt) vs. molting, and bout durations (>1 h and <1 h) (p-values <0.0001) (Fig. 2B). That some spontaneous activity persists during the molting period demonstrates that molting larvae retain the ability to move.

Increased consolidation of quiescence during the molting period could be due to a decreased drive to initiate and maintain behaviors such as feeding, an increased drive towards quiescence behavior, or both. It is also possible that sensory feed-back during food ingestion is required for maintenance of prolonged activity bouts. Slippage of the head capsule blocks the larval mouthparts, rendering larvae incapable of ingestion until ecdysis is completed. To determine whether food-deprivation can lead to a sudden increase in quiescence behavior, larvae were deprived of food beginning ~24 h prior to the predicted time of HCS, and behavior was recorded for 17 h. A typical quiescence profile for an individual food-deprived larva demonstrates that larvae are able to maintain long duration activity bouts in the absence of sensory feed-back from food (Fig. 2C). The average quiescence bout duration was ~10 min, and individual bouts never exceeded 1 h. Only ~4% of all bouts extended beyond 30 min. Average 1 h fractions of quiescence indicate that food-deprivation is accompanied by an initial increase in activity and for up to 17 h quiescence levels do not reach values observed for molting larvae. It is known that decreasing nutritional content of larval diet results in a compensatory increase in feeding behavior to maintain constant growth rates (Nijhout and Williams, 1974), and in flies and mammals, starvation results in a suppression of quiescence behaviors, such as sleep (McDonald and Keene, 2010). It is evident that the feeding homeostat is intact in the late 4th instar larvae used for this study. This suggests the interesting possibility that increased quiescence observed during the molt may signal an uncoupling of the feeding homeostat.

3.3. Larval quiescence is characterized by decreased sensory responsiveness and rapid reversibility

Quiescence is a general description that applies equally well to many very different states of arousal, including comatose, sleep-like, or resting states, intense vigilance, and paralysis. We have observed that molting larvae respond to noxious stimuli such as pinches to the dorsal horn or to contact with the tip of a hot soldering iron with robust defensive strikes. Additionally, molting larvae are able to right themselves when flipped onto their dorsal surfaces. These observations, coupled with detection of spontaneous activity during the molting period, suggest that peripherally,

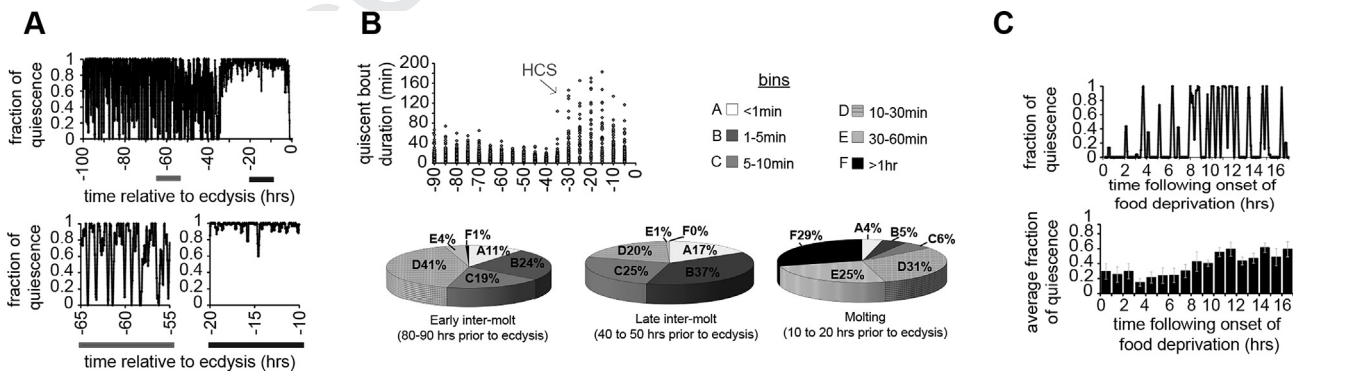

Fig. 2. Quiescence is more consolidated during the molt. (A) Sample rest-activity profile of an individual larva monitored under constant light conditions. Shown are fractions of quiescence in 10 min time windows shifted 10 s for each data point. Downward deflections represent increasing activity over time. (Time 0 = ecdysis to the 5th instar). (B) Durations of all quiescent bouts occurring throughout the 4th stage are plotted for each 5 h time-period set relative to ecdysis. (n = 8 larvae). Additionally, bouts occurring during early and late inter-molt, and during the mid-molt were assigned to one of 6 duration bins and for each stage the percentage of each bin is shown in pie graphs. (C) Fractions of quiescence in 10 min time windows shifted 10 s for each data point are shown for an individual larvae following the onset of food deprivation. Average 1 h fractions of quiescence are also shown. Time 0 = ~24 h prior to predicted time of head capsule slippage. (n = 6).

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the larval motor system can function normally during the molting period.

To test whether molting larvae can maintain activity for a more extended period of time and to compare inter-molt and molting responses to more complex environmental challenges, we performed behavioral assays that allowed for the observation of larval behavior during prolonged contact with a mildly arousing or noxious stimuli (Fig. 3A). Arenas were constructed so that placement on a water bath set to room temperature (~25 °C), 40 °C, or 50 °C resulted in an immediate dampening of the surface in contact with the larva. A ledge surrounding the periphery of the arena provided the possibility of escape from contact with the wet surface. Under "DRY" conditions, arenas were placed on the water bath, but a barrier prevented wetting of the contact surface. Since bouts of activity and quiescence are observed for inter-molt and molting larvae, we examined responses of larvae from both stages. To avoid differences due to size, only late inter-molt larvae were used for comparison. Larvae were monitored for 30 s prior to testing, and categorized as either aroused (active head movements for the 30 s prior to testing) or quiescent (immobile for the 30 s prior to testing). Spontaneous activity is rare for molting larvae, so to test responses of aroused molting larvae, we gently induced a single righting-reflex prior to monitoring, which was followed typically by movements of the head that resemble behavior observed for inter-molt larvae. Larvae that initiated locomotion during any monitoring session were excluded.

Following placement on the wet, room temperature bath, larvae of any stage or state of arousal exhibited behaviors that included searching, locomotion, and sometimes brief anterior and/or posterior thrashing movements. Although behaviors were qualitatively different compared to those observed under DRY conditions, almost all larvae in contact with the wet (room temperature) surface remained within the arena for the duration of the 10 min recording session (Fig. 3B). Inter-molt larvae that were quiescent prior to placement on the DRY bath maintained elevated quiescence levels (Fig. 3C), suggesting mechanical disturbances that might accompany gentle placement on the bath have minimal effect on arousal state. If aroused prior to placement on the DRY bath, inter-molt larvae maintain low quiescence levels typical for late inter-molt larvae (Figs. 3C and 1D) [*T*-test, t = 3.174 with 18 degrees of freedom. (P = 0.005), n = 10 10 quiescent and 10 control animals]. When placed on a wet, room temperature bath, quiescence levels resemble average inter-molt values independent of prior behavioral state (Fig. 3C) [T-test, t = 0.910 with 17 degrees of freedom. (P = 0.375), n = 10 quiescent, 9 aroused].

Molting larvae that are quiescent prior to placement on the DRY bath also display quiescence levels typical of mid-molting larvae (Figs. 3D and 1D), but they are reduced significantly for larvae that are aroused prior to testing [T-test, t = 4.717 with 18 degrees of freedom. (P ≤ 0.001), n = 10 quiescent, 10 aroused]. Both quiescent and aroused molting larvae show dramatically reduced quiescence levels when exposed to the wet surface, but in contrast to inter-molt larvae, quiescence is significantly reduced for aroused larvae (Fig. 3D) [t = 2.140 with 17 degrees of freedom. (P = 0.047), n = 10 quiescent, 9 aroused]. Surprisingly, when placed on a wet surface, activity levels of molting and inter-molt larvae do not differ significantly (Fig. 3E) [Quiescent animals: *T*-test, t = −0.910 with 18 degrees of freedom. (P = 0.375); Aroused animals: *T*-test, t = −0.0361 with 16

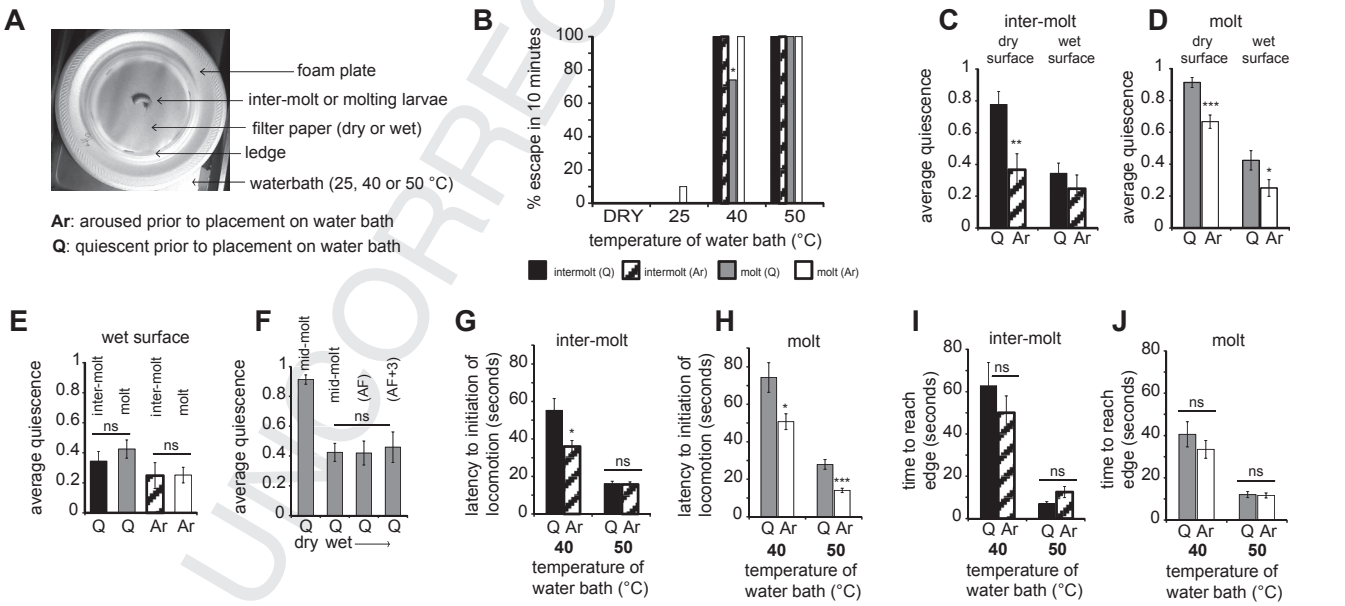

Fig. 3. Larval quiescence is a rapidly reversible state. (A) Schematic of behavior assay. Except under DRY conditions, the base of the arena is composed of filter paper that is dampened when placed on water baths set at 25, 40 or 50 °C. Under DRY conditions, the arena is placed on the room temperature bath and the un-cut foam plate prevents water contact. Larval behavior is recorded for a duration of 10 min or until the animal terminates contact with the water by climbing over a plastic ledge. "Ar" indicates that larvae displayed signs of arousal immediately prior to placement on the bath and "Q" indicates that larvae were quiescent prior to testing. (B) Shown are the percentages of larvae that escape the arena within a 10 min time-frame under DRY (control) or WET conditions at indicated temperatures. (C,D) Average quiescence levels of inter-molt (C) and molting (D) larvae monitored during for 10 min following placement on a DRY or 25 °C (room-temperature) bath. (E) Average quiescence levels during a 10 min contact with a wet room-temperature surface are compared for inter-molt and molting larvae. (F) Average 10 min quiescence level during contact with a wet room-temperature surface is compared for mid and late molting stages. AF = ~5–6 h, and AF+3 = 2–3 h prior to ecdysis. (G,H) For inter-molt (G) and molting (H) larvae, shown is the average time taken to initiate locomotion following contact with a water bath set at indicated temperatures. (I,J) Average time for inter-molt (I) and molting (J) larvae to reach the edge of the arena following initiation of locomotion on water baths set to indicated temperatures. (DRY and 25 °C, $n = 9–10$; 40 °C and 50 °C, $n = 17–24$). Tests for normality and equal variance were done with Kolmogorov–Smirnov and Levene Median tests, respectively. Significance is indicated as *p < 0.05, **p < 0.01, ***p < 0.001. All error bars are s.e.m.

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degrees of freedom. (P = 0.972)]. In these assays, molting larvae were staged so that testing occurred ~15 h prior to ecdysis. In order to test if larvae retain the ability to reverse their behavioral state throughout the molt, responses to a wet surface were also tested for larvae ~5-6 and ~2–3 h prior to ecdysis. There were no significant differences between quiescence levels measured during mid- and late-molting periods, which indicates that larvae are capable of reversing their behavioral state until late into the molt (Fig. 3F) [Kruskal–Wallis One Way Analysis of Variance on Ranks, H = 0.206 with 2 degrees of freedom. (P = 0.902), n = 10 mid-molt, 5 AF, 5 AF + 3] These results demonstrate that quiescence during both inter-molt and molting periods is a reversible state and that increased activity can be maintained by molting larvae at levels similar to inter-molt larvae for periods up to at least 10 min.

Rapid reversibility is one of the defining characteristics of a sleep-like state. The reversibility of sleep-like states is adaptive, in that it allows animals to respond to sudden, sometimes threatening changes in the environment, or to quickly renew normal behavior, such as feeding, when the sleep state is naturally terminated. In a sleeping animal, quiescence is also accompanied by reduced responsiveness to sensory stimuli, while reduction is reversed following arousal from the sleep-state. In *C* elegans larvae, response latencies to a noxious chemical stimulus are increased during the sleep-like molting state, and this reduced responsiveness is rapidly reversed upon arousal (Raizen et al., 2008). To test for similar differences in responsiveness in *M. sexta* larvae, we measured latency of responses to noxious thermal stimuli. When placed on a wet, 50 °C surface, larvae respond by initiating locomotion that results in a rapid linear displacement that culminates in contact with the peripheral ledge and subsequent escape from the wet surface within the 10 min recording session (Fig. 3B). This behavior contrasts with locomotion on the DRY or room-temperature baths where locomotion is often brief, non-linear and does not usually result in contact with the ledge.

When exposed to the 40 °C surface, inter-molt larvae also escape the arena, as do molting larvae that are aroused prior to testing; however, significantly fewer molting larvae escape the bath when testing is initiated during quiescence (Fig. 3B, Fisher Exact test P = 0.04633). This suggests that quiescence during the molting period is accompanied by a reduced responsiveness compared to inter-molt quiescence.

When placed on the 40 °C bath, both inter-molt and molting larvae take longer to initiate locomotion when in a quiescent state (Fig. 3G, H) [In G, Mann–Whitney Rank Sum Test, T = 254.000 (P = 0.037), n = 20 quiescent, 17 aroused; In H, *T*-test, t = 2.637 with 36 degrees of freedom. (P = 0.012), n = 19 quiescent, 19 aroused]. At 50 °C, latency to the initiation of locomotion was the same for inter-molt larvae regardless of pre-testing behavioral state [*T*-test, t = 0.219 with 41 degrees of freedom. (P = 0.828), n = 24 quiescent, 19 aroused], but molting larvae again displayed an increased latency when quiescent prior to testing [*T*-test, t = 5.110 with 32 degrees of freedom. (P ≤ 0.001), n = 16 quiescent, 18 aroused]. These results suggest that quiescence during both the inter-molt and molting periods is accompanied by a reduced sensory responsiveness that can be rapidly reversed following arousal. Differences across developmental state at 50 °C suggest that response thresholds are highest during molting quiescence.

If the quiescence state itself is rapidly reversible (as was suggested during the monitoring of activity on the room-temperature bath), then once aroused, behavior should be similar for animals regardless of the behavioral state prior to placement on the bath. This was confirmed when rates of locomotion were examined (Fig. 3I, J). [In I (40 °C), *T*-test, t = 0.906 with 35 degrees of freedom. (P = 0.371), n = 20 quiescent, 17 aroused, (50 °C), Mann–Whitney Rank Sum Test, T = 458.000 (P = 0.215), n = 23 quiescent, 19 aroused; In J (40 °C), Mann–Whitney Rank Sum Test, T = 309.000 (P = 0.497), n = 16 quiescent, 19 aroused, (50 °C), Mann–Whitney Rank Sum Test, T = 265.500 (P = 0.718), n = 15 quiescent, 18 aroused]. In addition, molting larvae locomote at rates comparable to those observed for inter-molt larvae. These results demonstrate that, although activity during the molt is dramatically reduced, the behavior of molting larva resembles that of inter-molt larvae.

3.4. Deprivation of quiescence during the inter-molt, but not the molt, results in a quiescence rebound

A characteristic feature of sleep-like states is homeostatic regulation. This property leads to compensation following a period of sleep deprivation, manifested as recovery sleep or "sleep-rebound". Such recovery sleep is more consolidated and longer in duration than normal sleep. Sleep rebound following deprivation is a generalized phenomenon observed in mammals, flies and worms (Huber et al., 2004; Raizen et al., 2008).

We subjected larvae to quiescence deprivation by flipping them every 12–14s, inducing a righting reflex. At the termination of quiescence-deprivation periods (Fig. 4A), time-lapse digital recordings were used to monitor behavior. Larval diet formed the base of the arenas. Weights recorded before and after quiescence-deprivation indicate that between righting responses, inter-molt larvae that were deprived for up to 6 h could feed normally on diet that formed the base of the arenas (Fig. S1C). Larvae were also deprived for 12 h, but since deprived animals did not gain weight at rates comparable to controls, those results were excluded from analysis.

When inter-molt larvae were deprived of quiescence for 3 h, the within animal likelihood ratio test based on a linear mixed model for repeated measures indicates that the fraction of quiescence for both inter-molt larvae and controls changes over time and that they are changing in different way (LRT = 14.65601, P = 0.0001). As can be seen, inter-molt larvae showed elevated fractions of quiescence compared to controls (Fig. 4B). The highest fraction (0.82) occurred during the first hour, when larval quiescence was increased ~44% above controls. Average quiescence during the first 6 h following deprivation was increased ~35% above controls. That levels are similar during the final hours of post-deprivation monitoring suggests that larvae are able to fully recover from the effects of deprivation.

After 6 h of deprivation, a within animal likelihood ratio test based on linear mixed model for repeated measures here also indicates that the fraction of quiescence for both inter-molt larvae and controls change over time and are changing in a different way (LRT = 11.64209, P = 0.0006). Again it can be seen that inter-molt larval quiescence is elevated compared to controls, with the greatest difference occurring during the first hour (51.6% increase) (Fig. 4B). During the first 6 h, quiescence is increased ~29% above controls. Although recovery of quiescence seems more robust during the first few hours for the shorter (3 h) deprivation time, the percent increase across the entire 24 h post-deprivation monitoring session indicates an overall increase of 13.5% for larvae deprived for 6 h compared to an overall 6.5% increase following 3 h deprivations. These results suggest that quiescence during the inter-molt period is regulated and as such is of some physiological importance to the animal. Reduced activity is unlikely to be the result of motor impairment; following a 3 h deprivation session, average rates of locomotion during exposure to a 40 °C water bath were similar for deprived (time to reach

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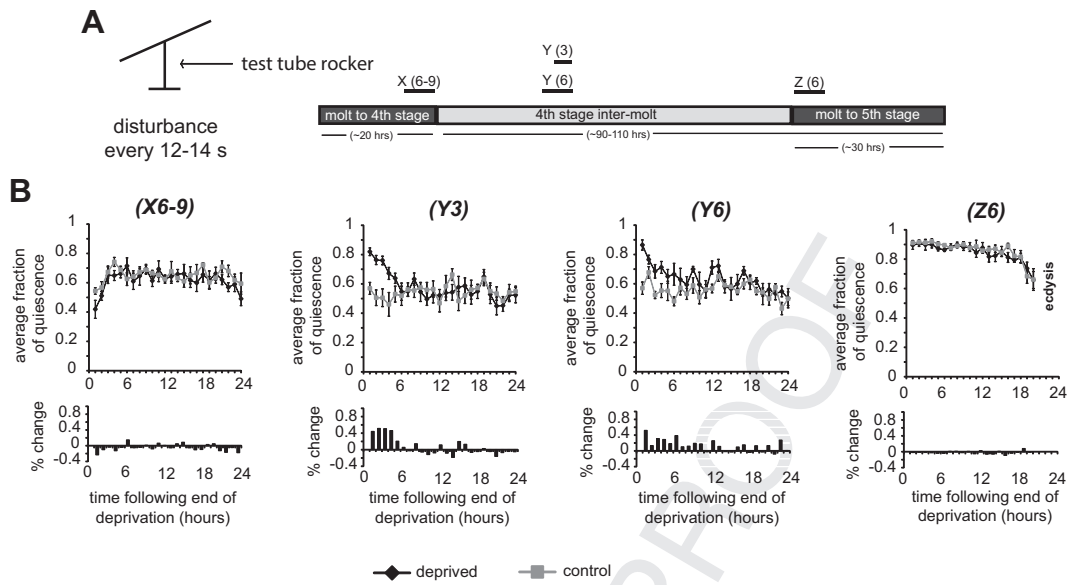

Fig. 4. Deprivation of quiescence during the inter-molt, but not the molt, is followed by quiescence-rebound. (A) Schematic of the semi-automatic deprivation protocol. Inter-molt or molting larvae were disturbed every 12–14 s for the indicated durations. X6-9 = deprived for 6–9 h during molt to 4th instar and recorded for 24 h during early inter-molt. Y3 and Y6 = deprived for 3 or 6 h during the inter-molt, and recorded for 24 h during inter-molt. Z6 = deprived for 6 h during early molting period and recorded ~20 h during mid-to late-molting period. The durations of the molt to the 4th instar, and the 4th larval stage are estimated from Fig. 1 (Time-line is not to scale) (B) Average 1 h fractions of quiescence following terminations of deprivation and percent (%) change relative to control. (n = 5–9 for each treatment and control).

edge = 23.8 ± 5.0 s.e.m. sec, n = 6) and control (30.0 ± 6.3 s.e.m. sec, n = 6) larvae.

In contrast, activity of molting larvae remains unchanged following quiescence deprivation (Fig. 4B). We established this using two groups of larvae: 3rd instars during the final 6–9 h of the molt and 4th instars during early molt hours. We found no significant change in quiescence levels during post-deprivation monitoring sessions in the early 4th instar inter-molt (LRT = 1.726041, P = 0.6312) or during the remainder of the molt to the 5th instar (LRT = 0.9559709, P = 0.62) compared to controls; however, a lack of observable rebound in the latter group may reflect a ceiling effect due to the high baseline quiescence levels. Deprivation during the molt also did not seem to affect developmental timing, as all larvae ecdysed to the 5th instar at predicted times relative to HCS. To verify lack of quiescence-rebound in molting larvae, they were manually deprived for 3 h following HCS during the early molt to the 5th instar. Animals were monitored continuously and when movement ceased for more than 30 s, they were gently flipped or tapped to induce movement. Following deprivation, larvae were allowed to rest for 15–30 min and responses to placement on a room temperature bath were tested (n = 6 deprived, 6 control). We were unable to compare fractions of quiescence during a 10 min exposure (previously shown to arouse molting larvae) because in contrast to non-deprived larvae, all but one of the larvae escaped the arena during the monitoring session. These results suggest that, in addition to lack of quiescence-rebound, responses of molting animals to arousing stimuli may actually be enhanced following deprivation.

Inter-molt larvae exhibit significant rebound following quiescence deprivation, suggesting that in terms of its behavioral properties, quiescence in these animals resembles the sleep-like states observed for flies and developing worms. That molting larvae fail to show quiescence-rebound suggests these animals are engaged in a distinct form of quiescence. As the amount of inter-molt quiescence is regulated, it is possible that processes dependent upon quiescent behavior may be somehow shut-down or altered during the molting process. In the adult fly, ecdysone signaling influences homeostatic regulation of sleep (Ishimoto and Kitamoto, 2010), although it is not known what effect this hormone has on arousal states or their regulation during the *Drosophila* larval stage. The molt-sleep may have evolved to provide the animal with a purely behavioral advantage during a time when feeding is absent and locomotion dangerous. It is also possible that molting quiescence cannot compensate for loss of inter-molt quiescence due to differences in underlying mechanisms (X6-9 group), or that the deprivation protocol used in this study was not sufficient to produce the sustained arousal of molting larvae. Rolling of larvae within deprivation chambers was observed to occur every 12–14 s for the duration of each session, but the time to complete a righting reflex is known to be brief (Adamo et al., 1997), and it was not possible to quantify the intervening behavior.

3.5. *M. sexta* larval brain transcriptome

Our behavioral data demonstrates that quiescence during the molt results from something other than impairment of motor function. We therefore asked whether reduced behavioral activity may be due to changes in the central nervous system that decrease general arousal state. In adult *D. melanogaster*, neural circuitry functioning in the brain contributes to modulation of arousal state (Cirelli and Bushey, 2008; Foltenyi et al., 2007; Griffith, 2013; Lebestky et al., 2009). Sites of multimodal sensory integration known to influence arousal state in adult insects also are present (although less well-developed) in the *M. sexta* larval brain (Homberg and Hildebrand, 1994; Kent and Hildebrand, 1987; Yack and Homberg, 2003). Neurotransmitters regulating arousal state in adult insects, including dopamine, serotonin, octopamine, acetylcholine, GABA and multiple neuropeptides, are also synthesized in larval brain and ventral nerve cord; it is likely that these signaling systems share conserved functions across developmental stages. Since the behavioral transition to the molt-sleep is so tightly correlated with developmental events driven by changes

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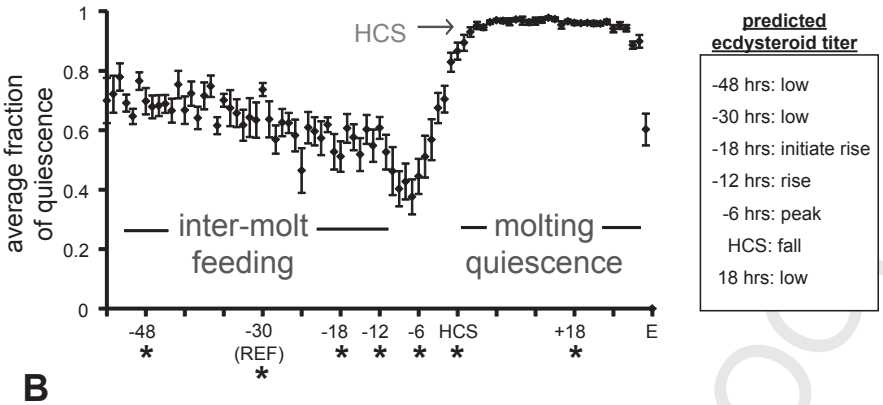

B

Library (staged relative to HCS)	library size (GB)	# sequences/ library	# genes mapped/ library	# differentially expressed/ library	UP (log_2fold ≥ 1)	DOWN (log_2fold ≤ 1)
-48 hrs	1.8	24452940	11841	1033	653	380
-30 hrs (reference)	1.9	25532383	11718	REF	REF	REF
-18 hrs	1.8	24398816	11778	904	521	383
-12 hrs	1.9	25498543	11785	1003	545	458
-6 hrs	2.3	31050934	11731	1467	729	738
HCS (time of)	2.0	27230965	11792	1908	1071	837
+18 hrs	1.7	22801779	11958	1601	1093	508

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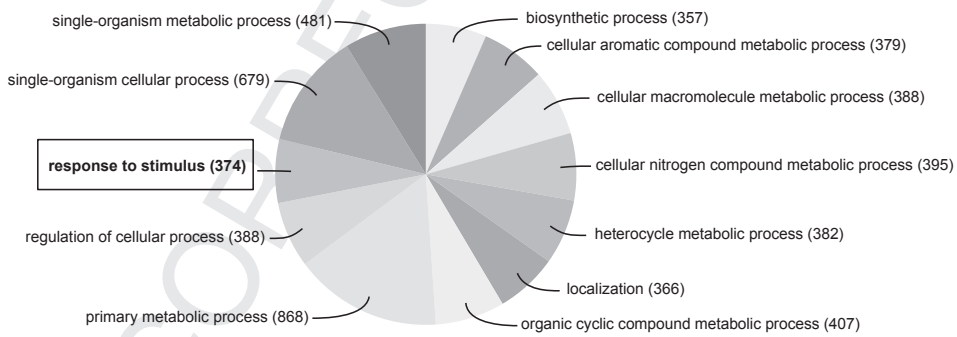

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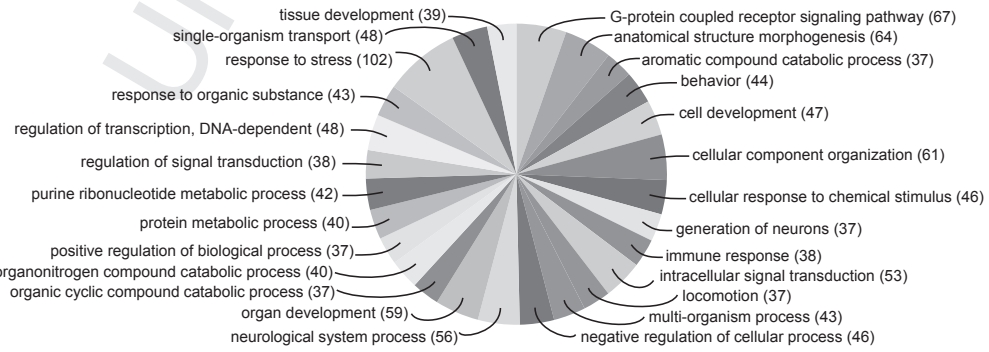

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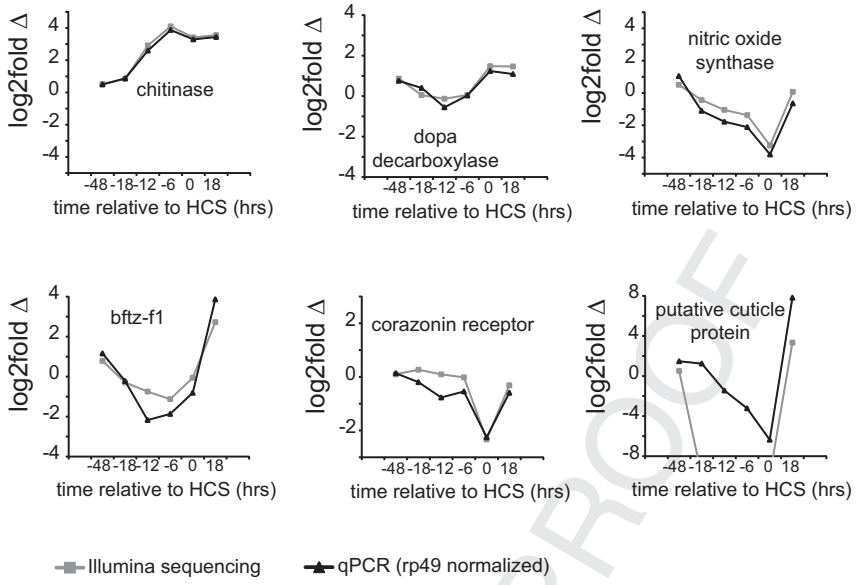

Fig. 6. qPCR validation. Expression profiles for 6 genes were validated using qPCR. All fold changes are relative to inter-molt reference stage (HCS −30 h). All $\log_2$fold changes reported for qPCR profiles are averages of at least 2 biological replicates, except for dopa decarboxylase (n = 1). Each biological replicate was run on an independent plate, and reactions were run in triplicate. For qPCR, ribosomal protein L32 was used as internal reference genes.

ecdysteroid levels, we speculated that neuroendocrine events might also correlate with changes in brain gene expression that could promote the onset and/or maintenance of molting quiescence. To investigate this possibility, we used Illumina RNAseq to compare the brain transcriptomes of larvae transitioning to (or within) the molt-sleep to transcriptomes of early and mid-inter-molt feeding larvae.

Larvae were staged relative to the time of head capsule slippage, and brains were dissected from larvae during the predicted rise, peak and fall of ecdysteroids (approach and transition to molt-sleep), during the middle period of the molt-sleep, and during the early, and mid-inter-molt feeding period (Fig. 5A). Pooled brain samples for each of the 7 stages were sequenced, and a total of ~13.4 GB of sequence data was generated across libraries (Fig. 5B). An average of 25,852,337 ± 2,662,408 (s.d.) reads were obtained for individual libraries. Reads were mapped using the most current version (Assembly v1.0) of the *M. sexta* genome (www.agripestbase.org/manduca/). Between 11 and 12 thousand genes were mapped in each library, and a total of 23,974 transcripts (isoforms for a predicted 13,335 unique genes) were mapped across all 7 time-points. As this is the first transcriptome profiling of the *M. sexta* larval brain, transcript ID and normalized counts are provided for all mapped transcripts, for each time-point (Table S1).

To predict which genes may be differentially expressed during development, normalized read counts were compared to the inter-molt feeding reference stage at HCS -30 h (Fig. 5A, B). Genes were considered differentially expressed if transcript level showed a greater than 2-fold increase or decrease across time-points. Known *M. sexta* transcripts were selected from the NCBI database (Fig S2A); staging and dissections were repeated and relative transcript levels were examined using qPCR. The close agreement between RNAseq and qPCR expression profiles served to validate our staging and sequencing methodologies, as well as use of the arbitrarily chosen cutoff value for differential expression (Fig. 6). Between 904 and 1908 genes were predicted as differentially expressed in any one stage, with maximal changes occurring at the time of HCS (Fig. 5B).

Across all stages, 3543 genes were predicted to be differentially expressed when compared to the inter-molt reference stage. For this set of genes, the most highly expressed isoform was selected and gene ontology (GO) terminologies and descriptions associated with the resulting set of sequences were obtained using the Blast2GO functional annotation tool (Conesa et al., 2005; Götz et al., 2008) (Table S1). When sequences were blastx searched against the nonredundant NCBI protein database, the species distribution of BLAST hits shows that the majority of hits are attributable to insect proteomes, and that most top-hits were to protein sequences belonging to the closely related species, *Bombyx mori* (Fig. S2A, B). The majority of differentially expressed sequences were mapped with GO terminologies associated with metabolic biological processes (Fig. 5C), and future investigation into these categories will require dissociation from processes related to tracheal cuticle formation. Three hundred and seventy-four genes were mapped with GO terminologies related to the parent term *response to stimulus*; and as this term is parent to many child terms associated with the regulation of behavior, we examined terminologies mapped to genes within this parent category. Within this set of genes, a

Fig. 5. The *Manduca sexta* larval brain transcriptome. (A) Brains were dissected at 7 time-points, staged relative to the time of head capsule slippage (HCS). Predicted ecdysteroid titers are based on radioimmunoassays that detect 20E (Langelan et al., 2000). (B) Illumina RNAseq was used to profile the brain transcriptome from each developmental time-point. Shown for each stage is the library size and the total number of unique genes that were mapped by ≥ 10 reads. Differential expression was determined using an inter-molt reference stage (30 h prior to head capsule slippage). For each library, the number of unique genes that showed an absolute fold change ≥ $\log_2$fold 1 and were mapped by ≥ 10 reads across reference and test conditions were reported as differentially expressed. (C,D) All transcripts that showed differential expression during at least one developmental time point and were mapped by at least 10 reads in at least one stage were isolated and filtered so that only the most highly expressed isoform was included. The resulting list of 3543 differentially expressed genes was then annotated in BLAST2GO. In (C) is shown the sequence distribution by GO terminologies annotated under the parent term biological process (sequence filter 354). In (D), sequence distribution by GO terminologies mapped to the 374 sequences associated with the parent term "response to stimulus" are shown (sequence filter = 37).

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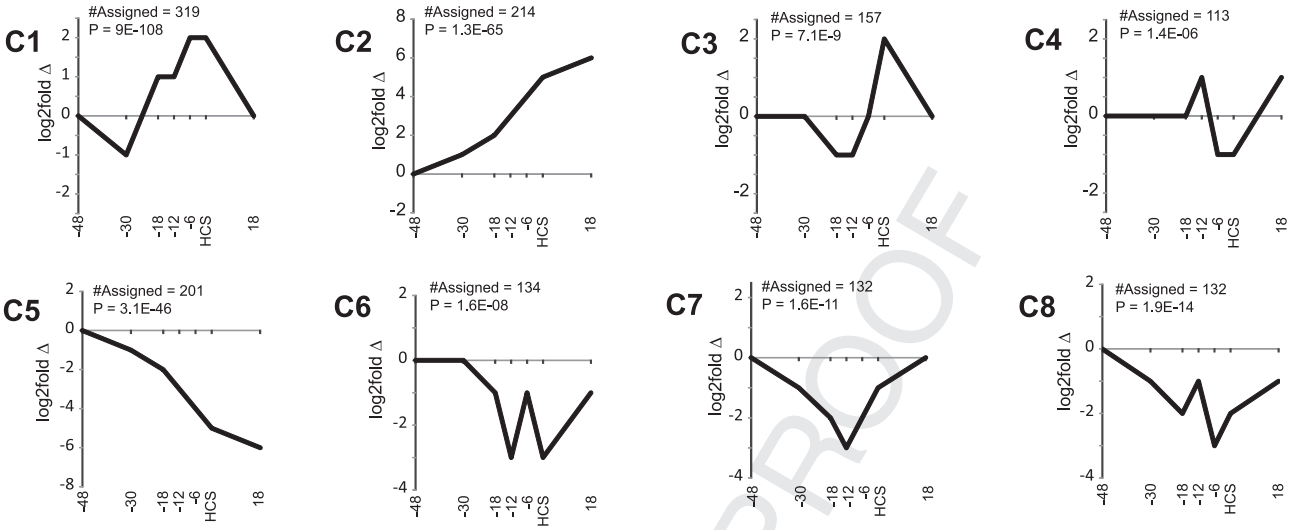

B

G-protein coupled receptors

		(-)48	(-)18	(-)12	(-)6	HCS	(+)18	
(C1)	Msex2.01193-RA	0.3	0.5	0.8	1.2	0.9	0.0	amine receptor/moody
(C1)	Msex2.12222-RA	0.8	0.4	0.8	1.1	1.0	0.3	GPCR/frizzled
(C4)	Msex2.04332-RB	0.0	-0.6	-0.6	-1.1	-2.0	0.1	nucleotide receptor/adenosine receptor
(C5)	Msex2.11354-RA	-0.3	-0.5	-1.3	-1.4	-1.3	-1.3	rhodopsin-like neuropeptide receptor/neuropeptide receptor A34
(C5)	Msex2.08925-RA	0.0	-0.5	-1.0	-1.3	-1.3	-0.7	amine receptor/serotonin receptor
(C5)	Msex2.07613-RA	0.2	-0.3	-0.7	-1.1	-2.0	-0.9	GPCR/atrial natriuretic peptide receptor 1-like
(C5)	Msex2.01275-RA	-0.4	-0.7	-0.8	-0.5	-1.2	-1.4	rhodopsin-like neuropeptide receptor/myosuppressin receptors
(C5)	Msex2.02062-RA	0.5	0.0	-0.3	-0.5	-1.2	-0.6	GPCR/melatonin receptor-like
(C5)	Msex2.02917-RB	0.2	-0.4	-0.7	-0.9	-0.9	-1.1	rhodopsin-like neuropeptide receptors/tachykinin receptor
(C6)	Msex2.11502-RA	0.4	0.1	-0.8	-0.4	-1.5	-0.5	rhodopsin-like neuropeptide receptor/neuropeptide receptor A2
(C7)	Msex2.02255-RA	0.4	-1.1	-1.6	-2.4	-1.3	0.8	GPCR/methuselah-like
(C7)	Msex2.04092-RG	0.3	-0.3	-0.8	-0.7	-0.3	1.1	GPCR/chemosensory
(C8)	Msex2.10524-RB	0.2	-0.5	-0.2	-1.6	-0.9	-0.3	rhodopsin-like neuropeptide receptors/neuropeptide Y receptor
(C8)	Msex2.09313-RB	0.0	-0.3	-0.6	-1.3	-0.6	-0.4	amine receptors/dopamine receptor-2

cyclic nucleotide signalling pathways

		(-)48	(-)18	(-)12	(-)6	HCS	(+)18	
(C4)	Msex2.04999-RB	0.0	-0.1	-0.6	-1.0	-1.5	0.5	guanylate cyclase
(C5)	Msex2.04545-RA	0.3	-0.2	0.0	-2.1	-1.9	-1.5	cAMP-dependent protein kinase
(C5)	Msex2.06671-RB	-0.2	-0.5	-0.5	-1.3	-1.3	-0.9	guanylate cyclase/atrial natriuretic peptide receptor
(C5)	Msex2.12762-RA	-0.1	-0.3	-0.3	-0.5	-2.2	-1.8	adenylate cyclase
(C7)	Msex2.02444-RA	0.2	-0.4	-0.9	-1.5	-0.7	1.3	soluble guanylate cyclase

neurotransmitters / neuropeptides

		(-)48	(-)18	(-)12	(-)6	HCS	(+)18	
(C1)	Msex2.04985-RA	0.4	0.3	0.4	0.9	1.2	0.2	atrial natriuretic peptide-converting enzyme
(C5)	Msex2.05880-RB	1.4	-0.9	-1.3	-1.4	-1.2	-1.3	adipokinetic hormone precursor
(C6)	Msex2.06762-RA	0.5	-0.4	-1.0	-1.4	-3.2	0.1	nitric oxide synthase
(C7)	Msex2.00239-RB	0.5	-1.0	-2.0	-3.2	0.4	-0.4	acetylcholinesterase

ligand-gated ion channels / ion channels

		(-)48	(-)18	(-)12	(-)6	HCS	(+)18	
(C1)	Msex2.05451-RF	0.1	0.2	0.5	1.0	1.2	-0.2	voltage-dependent calcium channel
(C2)	Msex2.02703-RA	0.0	1.2	2.0	2.1	1.6	4.7	neurotransmitter gated ion channel/glycine receptor
(C3)	Msex2.00019-RA	-0.1	0.8	0.5	0.2	2.9	1.2	transient receptor potential channel
(C3)	Msex2.01924-RC	0.2	0.0	-0.2	0.0	1.5	0.4	chloride channel
(C3)	Msex2.04551-RA	0.4	0.3	0.4	0.6	1.4	0.7	transient receptor potential channel
(C5)	Msex2.14197-RB	-0.1	-0.3	-0.5	-1.1	-1.7	-1.5	potassium channel
(C5)	Msex2.02070-RA	0.7	-0.1	-0.6	-0.9	-1.3	-0.5	transient receptor potential channel
(C6)	Msex2.09581-RA	-0.2	-0.4	-0.5	-1.0	-1.9	-0.4	neurotransmitter gated ion channel/glycine receptor
(C8)	Msex2.04543-RA	0.3	-0.4	-0.4	-1.0	-0.5	-0.3	potassium channel

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diverse range of biological processes linked to behavior emerges, including response to stress, regulation of signal transduction, G-protein coupled signaling pathway and locomotion (Fig. 5D).

3.6. Cluster analysis

Since a large number of differentially expressed genes were annotated with terminologies associated with signal transduction, we probed this subset to identify gene products that might either promote quiescence or reduce behavioral drive during the molt. To narrow our search, we used the software Short Time-series Expression Miner (STEM) to cluster groups of genes with similar expression profiles (Ernst and Bar-Joseph, 2006). Twelve significant clusters (C1–C12) were identified using this method (Table S2). We assumed that genes important for promotion and maintenance of molt-sleep would show expression changes specifically during the approach to and around the time of the behavioral transition. Eight of the profiles (C1–C8) were assigned genes with relatively stable expression across the two sampled inter-molt time-points, but showed up- or down-regulation during the approach to or onset of molt-sleep (Fig. 7A). For genes in these clusters, BLAST2GO annotations were searched for descriptions and GO terminologies related to signal transduction. Within the lists of C1–C8 transcripts, we identified G-protein coupled receptors (GPCRs), elements of cyclic nucleotide signaling pathways, neurotransmitters and neuropeptides, and ion channels (Table S2, Fig. 7B). Gene names and functional descriptions were ultimately assigned by identifying putative orthologs in NCBI, SilkDB and Flybase. In general, expression of GPCRs and components of cyclic nucleotide signaling pathways was depressed during the approach to and maintenance of molt-sleep (Fig. 7B). Down-regulation of these genes implies their importance in promotion of inter-molt-stage behaviors. The NPY-like system, for example, is known to promote feeding behavior in the *Drosophila* larval stages (Wu et al., 2003). Three up-regulated genes (identified from C1) include a putative atrial natriuretic peptide (ANP) converting-enzyme (Msex2.04985), and two putative GPCRs, Msex2.01193 Msex2.12222, predicted as orthologous to moody-like and frizzled-like receptors, respectively. Neither ANP signaling nor either of the two receptor subfamilies has been previously implicated in arousal state modulation. Moody-like receptors are expressed in glial cells and are important to maintenance of the blood brain barrier (Hatan et al., 2011) and it is possible that strengthening of this barrier is necessary during a time of peripheral hormonal flux.

Nine genes were identified with annotations predicting their functions as ion channels or specifically ligand-gated ion channels (Fig 7B). In contrast to the above-described categories, over half of these genes are assigned to up-regulated clusters C1–C3. Up-regulated genes include a putative voltage-dependent calcium channel (Msex2.05451), two putative chloride channels (Msex2.02703 and Msex2.01924), and two putative transient receptor potential channels (Msex2.00019 and Msex2.04551). In this same functional category, a putative transient receptor potential channel (Msex2.02070) and a chloride channel (Msex2.09581) are down-regulated, as are two putative potassium channels (Msex2.14197 and Msex2.04543). The effects on arousal state of changes in neuronal ionic conductance are likely to be circuit-specific. GABA, for example, promotes sleep in flies and mammals through its actions on ligand-gated chloride channels expressed in wake-promoting circuitry (Chung et al., 2009; Siegel, 2004). Future studies can aim to investigate the location of channel expression, and their potential roles in either the promotion of quiescence or depression of wake-promoting circuitries.

4. Conclusions

Although it has long been recognized that the larval molts of *M. sexta* are associated with an increased level of quiescence, this study is, to our knowledge, the first to characterize molt-sleep behavior. Molting quiescence is a state of reduced responsiveness that is rapidly reversible, resembling the sleep-like state of adult insects. We provide evidence suggesting that homeostatic regulation of both feeding and quiescence are depressed during molt-sleep. The tight correlation of molt-sleep onset with the predicted surge of molting hormones suggests either a direct or indirect programming of molting quiescence via the neuroendocrine system. An unexpected finding is that molting larvae retain the ability to locomote and to respond to noxious stimuli with a level of robustness similar to feeding-stage larvae. We propose that molting-quiescence confers a selective advantage in the natural environment. The molt is accompanied by morphological changes that for the duration of the molt-sleep prevent the ingestion of food. Decreased behavioral drive coupled with retention of ability to respond to a changing environment would both conserve energy and allow for protection against threatening conditions, thereby increasing viability. The long duration of its inter-molt and molting periods, coupled with predictability of behavioral transitions in relation to hormonal changes, suggests that *M. sexta* may serve as a useful model system to study regulation of arousal states and homeostatic control mechanisms by both endocrine and nervous systems.

As a first step in exploring possible neural mechanisms regulating larval arousal state, we sequenced the brain transcriptome at 7 developmental time-points to examine changes in transcript identity and level during the transition to molt-sleep. Over 13 thousand genes were mapped by sequences from brains taken across the 4th larval stage, and 3543 genes were predicted as differentially expressed during at least one developmental time-point. By exploring a small subset of genes associated with signal transduction, we found that the transition to molt-sleep is accompanied by a general depression of GPCR expression and a down-regulation of genes connected to cyclic nucleotide signaling. In contrast, several ion channels were up-regulated during the transition to the molt-sleep, including a voltage-dependent calcium channel, two putative chloride channels and two transient receptor potential channels. The conservation across insects of many arousal-related brain regions and neurotransmitter systems provide future opportunities to use more genetically tractable organisms, such as *D. melanogaster*, to explore the possible role of these and other genes differentially expressed during molt-sleep in modulation of the insect arousal state.

Fig. 7. Short Time-series Expression Miner (STEM) clusters. (A) The 3543 genes predicted to be differentially expressed (Fig. 6C; Supplemental Fig. 2) were further analyzed using the STEM algorithm. (A) Shown are 8 of the 12 identified significant time-series expression profiles (clusters C1–C8). (Bonferonni FDR <0.05). Counts were log normalized to the earliest developmental stage (HCS -48hrs) and $\log_2$fold changes are plotted relative to time of development relative to head capsule slippage (HCS). The number of genes assigned to each cluster and p-value are in the upper left corner of each profile. (B) Blast2GO annotations for all genes in Clusters C1–C8 were searched for descriptions and GO terminologies associated with functional categories related to signal transduction. Transcripts predicted to be associated with each of the specified categories are listed under each heading and ordered according to cluster. Sequence ID (left) and predicted description (right) are given along with $\log_2$fold changes relative to inter-molt reference stage (HCS -30hrs). Black boxes indicate >2-fold up-regulation ($\log_2$fold > 1), and white boxes indicate >2-fold ($\log_2$fold < −1) down-regulation for the indicated stage. Under the category *G-protein coupled receptors*, a description in black indicates that the top Bombyx hit was identified and described in Fan et al. (2010).

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Author contributions

DM designed and performed the experiments, analyzed the data and wrote the manuscript. PA performed the sequence alignment and DEseq analysis. JH assisted with qPCR primer design and colony rearing. XC helped analyzing the data. MA managed the project and helped edit the manuscript.

Uncited reference

Q4 Sawin et al., 1994.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ibmb.2015.01.012.

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